THE ROYAL SOCIETY

Published online 27 June 2002

In vitro uses of biological cryoprotectants

Peter J. Lillford* **and Chris B. Holt**

Colworth Laboratory, Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK

Ice can be anything from a highly destructive agent in agriculture to a useful building material. Established industries are based on the known rules of physics and chemistry which allow some control of amounts of ice or ice crystal geometry. However, organisms have much more subtle requirements to maintain their delicate internal structure if they are to survive freezing. As a result they have selected specific molecules for freezing-point depression, osmotic regulation, ice nucleation and crystal growth inhibition. All these active species may have potential commercial use once they are identified, understood and produced at economic scales.

We examine the progress made so far in extending biological subtlety into commercial processes, and look for prospects for further innovation.

Keywords: cryoprotectants; antifreezes; nucleators; glasses

1. INTRODUCTION

The range of industries involved in controlling freezing processes or the properties of ice are enormously varied in their application and technological sophistication, but they all have to control the same phenomena: the amount of ice, its size, shape and location. In figure 1, these industries are summarized, showing something about their scale, profit margins, the systems in which ice is being formed and therefore the sophistication of their processes.

It is not too surprising after inspecting figure 1 that the technology develops by input from disciplines ranging from meteorology through physical chemistry and engineering to cell and molecular biology; and the technical literature historically shows feedforward and feedback across the sciences and the industries. Some examples of the 'heroes' of technology are

(i) Francois Raoult, who systematized the effects of dissolved solutes on the freezing of water;
(ii) Clarence Birdseye, who developed the first large scale commercial food freezer (Birdseye & Hall 1931); and
(iii) *Rana sylvatica*, an American tree frog who can survive freezing and thawing with remarkably little damage.

Unfortunately, the last contributor does not publish his technical secrets in the open scientific or patent literature and it is left to others to discover the sophistication of his methods (Storey & Storey 1988).

We will examine the various industries' approach to their common issues of controlling the amount, nucleation and growth of ice and how knowledge can be transferred between them. This allows some projection of how future technologies might develop.

We begin at the 'easy' end, with control of amounts of ice and the use of dissolved solutes.

2. ICE MINIMIZATION

Raoult's law (figure 2) describes the surprising fact that freezing-point depression depends mostly on the size of the solute rather than its chemistry, or that at any given temperature the amount of ice is most efficiently reduced by adding more small molecules.

The constraints on which additives can be used industrially are highly system dependent. Common salt is used on roads because it is cheap. Ethylene glycol works at lower temperatures and does not rot radiators but is toxic to humans. The range of frozen confectionery products depends extensively for its textural qualities on the controlled use of polyols, predominantly sugars. Despite their relative inefficiency relative to glucose, lactose, sucrose and glucose syrups are used because of low cost. There are innumerable patents in the food literature describing combinations of small sugars and mechanical processing to produce 'soft' ice-cream, which requires low ice content and small crystals (Cole *et al.* 1983). The former is the predominant requirement, but the inability of small solutes to limit ice crystal growth and sintering still makes long-life soft ice a challenging target.

Amino acids are too expensive at the level required for ice reduction in foods and would produce a significant flavour change, although the 'fortified' ice-cream may be a future marketing position.

As soon as animal or vegetable tissue is to be frozen with minimal damage, then additional major constraints must be considered. For food purposes, the maintenance of osmoregulation is not required and functional membranes and enzymes need not be preserved, but the recovery of cellular architecture after freezing and thawing must occur or texture will be radically different. Commercial

* Author for correspondence (pl8@york.ac.uk).

One contribution of 15 to a Discussion Meeting Issue 'Coping with the cold: the molecular and structural biology of cold stress survivors'.

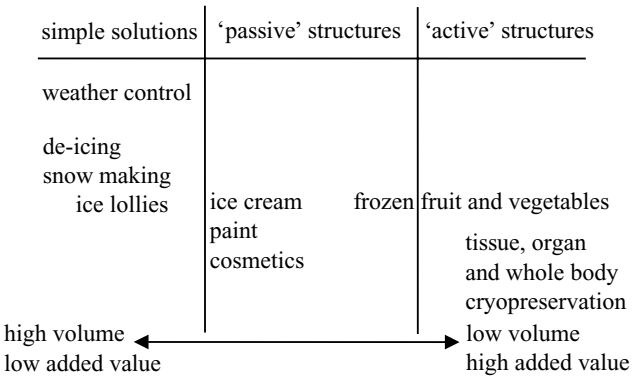

Figure 1. Industries with interests in controlling freezing.

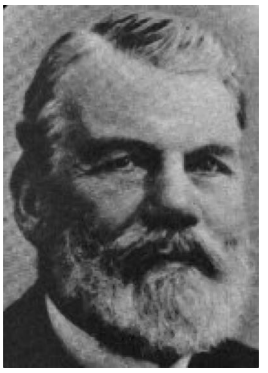

Figure 2. François-Marie Raoult (1830–1901). Raoult's law: the vapour pressure of a solvent in an ideal solution decreases as its mole fraction decreases.

freezing almost always causes some damage as ice crystals grow both inter- and intracellularly. On thawing, the damage is not reversed; cells remain collapsed, resulting in 'thaw drip' from meat and fish, and the mushy texture of many fruits and vegetables (figures 3 and 4). It is self-evident that any of the tools of tissue or organ preservation are immediately relevant to solving this problem. This is why there is interest from the food industry in the mechanisms permitting freeze tolerance in natural systems, and in the techniques of tissue cryopreservation.

In the meantime, the techniques of freeze avoidance are helpful. By permeating tissue with edible small molecules, ice content and subsequent damage are reduced, but the resultant textures are those of candied fruits or vegetables rather than the natural product. Because membrane viability is not necessary, preblanched tissue is frequently used, permitting more rapid mass transfer of the small solutes into the cellular structures.

The alternative route to remove ice crystallization is rapid cooling into the glassed state. Certainly this can eliminate structural damage during freezing, which is the basis of cryomicroscopy. But mass transfer rates limit the sample size in which this can be achieved and thawing is never rapid enough to prevent crystallization from causing extensive damage.

Variable-rate freezing produces some advantages for food quality. Here, initial cooling down to the freezing point is slow, minimizing thermal gradients across the sample and permitting some undercooling within the cells. The presence of small solutes is advantageous because the degree of supercooling is increased by more than the reduction in freezing point. Then cooling is accelerated in an attempt to produce many nuclei throughout the sample. Intracellular freezing is advantageous because gross structure is maintained even though metabolic processes and membrane compartmentalization are destroyed. Unfortunately, nucleation is variable between samples and not easily controlled, the equipment requires greater capital investment and throughput is lower. The best frozen products are still those whose structure and composition are resistant to, or recover from, the presence of ice.

Freeze-tolerant and freeze-avoiding organisms require much more sophisticated protection, but appear to use Raoult's law, producing small molecules intracellularly. For example, amino acids, glycerol, sorbitol and even ethylene glycol are pressed into service (Storey & Storey 1988; Duman *et al.* 1991; Chen *et al.* 1995). However, it is intriguing to note how a common set of small molecules are selected when according to Raoult this is not necessary. Are these molecules selected for their particular ability to regulate ice, or do they have other functions in structure protection? If so, they will be industrially important. Are they simply the least damaging metabolites left behind by thermally modified metabolic processes? Considerable effort has been expended in attempting to answer these questions, but complete understanding and consensus have not been achieved.

First, we must recognize that Raoult's law is not adequate to describe the freezing process at high solute concentrations or low temperatures. A second restriction to ice growth occurs when the glass transition point of the solute is reached (figure 5).

This identifies a composition at which the ice growth effectively stops and is a kinetic restriction rather than a thermodynamic phase transition. This state has been extensively studied recently and certainly can play a part in industrial processes (Blanshard & Lillford 1993). The figure shows the glass transition line for a small sugar and a polysaccharide. Note that the latter is most effective in elevating the temperature of glassing but permits the formulation of more ice than the small molecule before glassing occurs. Despite much debate, it is now recognized that glassing of comparable molecular weight sugars occurs at similar temperatures, so that like Raoult's law, size matters rather than molecular configuration in determining glass transitions. It is probable that natural systems exploit this physical state, and this will be discussed for specific cases.

3. TREHALOSE VERSUS OTHER SUGARS

This non-reducing sugar is produced by yeasts and insects in copious amounts (Duman *et al.* 1991). Its use as a cryoprotectant in commercial systems has been patented widely (Roser 1987, 1989; Tunnacliffe *et al.* 1998). However, whether its properties are in any way unique is still contentious. In simple systems it shows little more capability of protecting protein structure against freezing than other simple sugars. In our hands, sucrose and other higher-molecular-weight non-reducing sugars such as raffinose and stachyose are equally effective. Patents on the use of most common polyols have also been granted (Franks *et al.* 1996). Arguments that trehalose is able to glass at higher temperature than comparable dissacharides

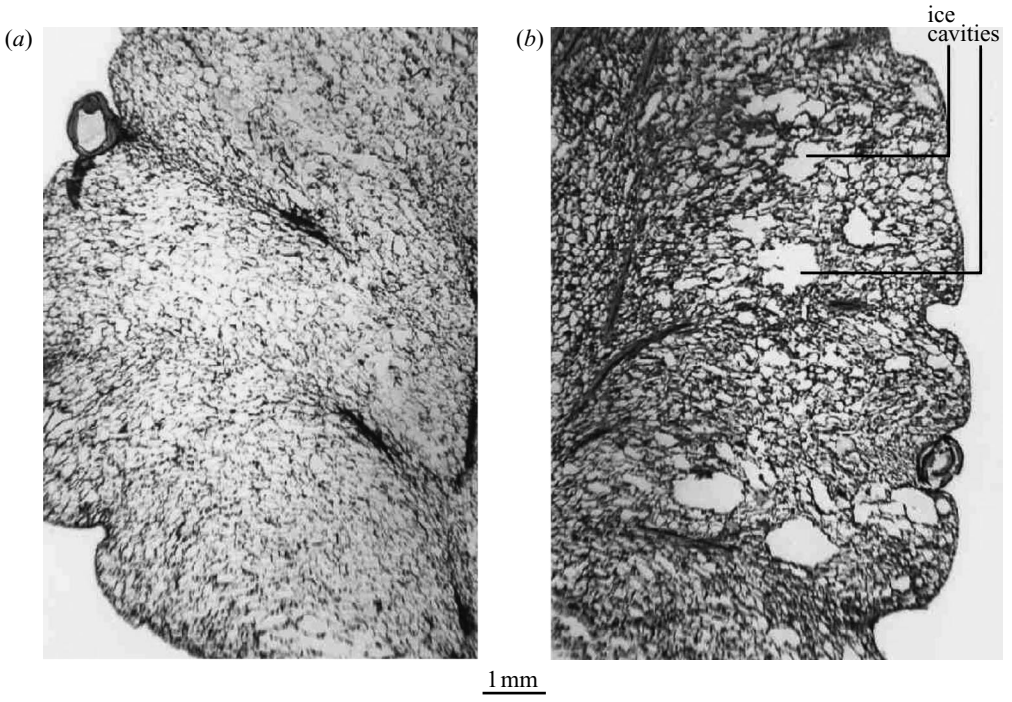

Figure 3. Effects of freezing/thawing on strawberry. (*a*) Fresh; (*b*) frozen/thawed.

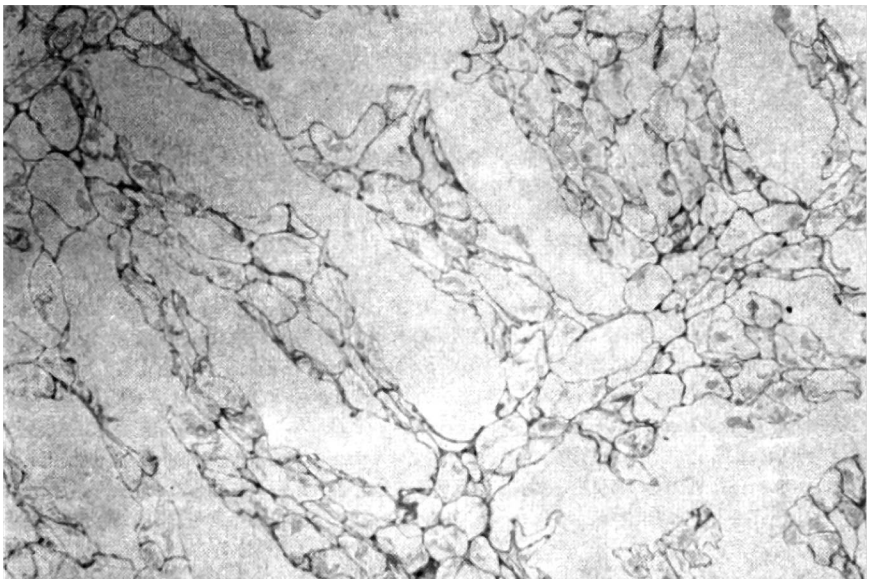

Figure 4. Thin section of frozen/thawed carrot tissue (large voids are regions of thawed ice).

are unfounded. Experiments are reported which show that it may have uniqueness in protecting membrane structures (Rudolph *et al.* (1986), although the authors report similar effects from galactose.) Even though these results relate to man-made systems rather than intact biological cells, they may have commercial value. In natural systems, so many other metabolites are modified simultaneously during cold acclimation that it is difficult to assign the absolute importance of trehalose.

Plants tend to accumulate other non-reducing sugars, particularly sucrose, and many are freeze tolerant. However, their need to simultaneously adjust cell wall structures (Weiser *et al.* 1990) and membrane composition (Uemura & Steponkus 1997) appears mandatory.

The accumulated sugars in all organisms are predominantly non-reducing. There may be some advantage in these non-reactive forms, as their local concentration in the frozen state will be very high (more than 60% wt : wt). However, no significant evidence of Maillard condensation between reducing sugars and amine groups has been demonstrated. By contrast, commercial products (and the frog) utilize glucose without signs of significant browning or protein modification (Storey & Storey 1988).

4. AMINO ACIDS

Natural systems under freezing and drought stress also accumulate amino acids which can contribute to freezing-

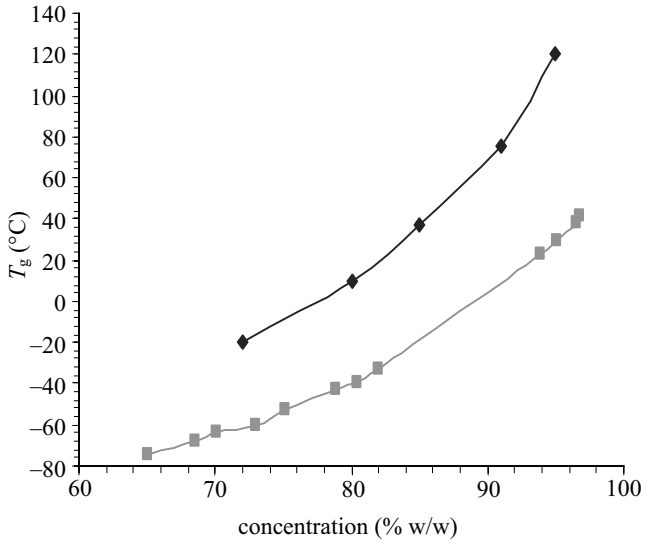

Figure 5. Relationships between the glass transition temperature (T_g), concentration and molecular weight (diamonds, pullulan; squares, sucrose). (Source: Ablett *et al.* (1992) and Blanshard & Lillford (1993).)

point reduction and osmoregulation. Though increased levels of many amino acids have been demonstrated during cold acclimation, particular increases in proline, glycine and betaine appear to be associated with these stresses. Is there any significant action by these particular molecules or do they simply represent accumulation of metabolic intermediates with no particular function other than that they cause no harm? We have found that most amino-acids anion are at the 'structure-making' end of the Hoffmeister series and protect proteins *in vitro* against thermal denaturation and drying damage, but proline is not significantly better than others and is lower in the series than glutamate. It has been argued that proline has membrane-stabilizing effects that are larger than other amino acids (Rudolph *et al.* 1986), and there is an intriguing report from the polymer literature showing that proline is a significantly better plasticizer of polysaccharide (starch) polymers than would be expected (table 1; Stein *et al.* 1999). More systematic studies on the action of amino acids are required before significant special opportunities can emerge.

5. ICE CRYSTAL GROWTH AND RECRYSTALLIZATION

(a) *Polymeric solutes: non-specific effects*

Dissolved polymers can significantly reduce ice crystal growth rates. The general mechanism appears to be not molecularly specific but is caused by the reduced diffusion rates of macromolecules away from the growing ice interface. There are special cases in which the increased polymer concentration in the unfrozen phase can cause 'cryogelation': the formation of a three-dimensional network with an effectively zero diffusion coefficient. Patents for control of ice and clathrate ice growth and formation have been granted and the technology is widely used commercially (Finney 1976; Duncum *et al.* 1994). Because biopolymers exhibit this phenomenon, it is surprising that this mechanism has not been widely reported in freeze-tolerant organisms. Perhaps the modification of cell wall polymers during cold adaptation can contribute to limiting crystal growth.

As mentioned previously, dissolved polymers form glasses readily once freeze concentrated. Below this temperature no further ice forms, and crystal growth rates and sintering of ice are kinetically slowed to almost zero. To achieve this condition, at the highest sub-zero temperatures, small solutes should be removed, but this increases the ice content. Thus, the use of glassing phenomena is a balance between damage caused by the amount of ice present and the temperature at which it is stabilized against growth and/or recrystallization.

(b) *Polymeric solutes: specific effects*

(i) *Antifreezes*

The non-colligative effects of certain natural polymers on apparent freezing-point depression have been widely reviewed (Lillford & Holt 1994) and identify them as 'antifreezes'. It is important to recognize that in this function of freezing-point depression they are inefficient relative to cheaper, small solutes. Of much more interest commercially is their performance after ice is nucleated or

Table 1. Amino acids as plasticizers. (Source: Stein *et al.* (1999).)

	% elongation at break	plasticizer effectiveness
1 1-piperidinepropionic acid	290	good
2 L-proline	149	
3 *N*,*N*-dimethylglycine	83	moderate
4 4-aminobutyric acid	61	
5 sarcosine	59	
6 β-alanine	52	
7 betaine	42	non-effective
8 L-alanine	27	
9 glycine	19	poor
10 L-isoleucine	17	
11 L-leucine	16	
11 L-valine	16	

under conditions where ice crystals would normally grow and sinter. Both freeze-avoiding and freeze-tolerant organisms produce antifreezes and the proteins or glycoproteins used show little structural homology but they all exhibit similar phenomena when ice form.

First, on cooling, the crystals formed are smaller, more uniform and often of different shape to those in a simple solute system. Smaller size probably relates to deeper supercooling, implying that antifreezes are preventing heterogeneous nuclei from growing rapidly, i.e. they are 'anti-nucleators'. Second, independently of the cooling regime, the formed crystals do not grow or accrete at temperatures where this would normally occur, i.e. they are 'growth inhibitors'. They therefore provide the enormous technological potential of controlling ice crystal size, shape and stability in any industrial process. They are so efficient that their presence in tissue is at a relatively low concentration, so that the cost of their extraction from natural sources prohibits their use in anything but applications with very high added value. However, recent advances in biotechnology and control of heterologous gene expression mean that material is potentially accessible in kilogram quantities, although as ingredients they will remain expensive (Warren *et al.* 1990). Nonetheless, small-scale demonstrations of industrial potential and a portfolio of patents are developing almost daily. Some examples are given below.

(i) Plant protection: reduced ice content (Cutler *et al.* 1989) and reduced recrystallization (Hightower *et al.* 1991).
(ii) Fish: prevention of freezing injury to live salmon by trangenic expression (Hew *et al.* 1995).
(iii) Meat: drip reduction by soaking in AFPs (Payne *et al.* 1994).
(iv) Ice-cream: crystal size reduction (Clemmings *et al.* 1996) and shape control (Needham *et al.* 1998).
(v) Cryopreservation: addition to pig oocytes (Rubinsky *et al.* 1992).
(vi) Cryosurgery: enhanced local cell damage by AFP perfusion (Koushafar *et al.* 1997).
(vii) Air conditioning: improved low temperature circulation (Grandum & Nakagomi 1997).

As commercial quantities of AFPs become available, this list can only increase and despite concerns of European consumers with respect to genetic manipulation, many applications will be converted to this practice.

6. ICE NUCLEATION

A complete review of the physical chemistry of ice nucleation in biological systems and their application is available elsewhere (Lee *et al.* 1995). Unlike antifreezes, a natural source in commercial quantity and cost is available in the form of cells and fragments of *Pseudomonas syringae*. This means that large-scale low-added-value uses are already in practice such as snow making for ski slopes (Woerpel 1980) and even road and bridge constructions in ice in the Arctic (Owen *et al.* 1987).

Although cheap, these preparations are not allowed in food and are dispersions of insoluble cell debris with the active protein decorating the surface. The development of soluble nucleators allowed in food is a future target because this will increase access to higher margin industries. The second and more difficult target is to control their molecular size.

As nucleation requires the provision of a nucleation site of critical radius at any given temperature, the dominant nuclei in a population of sizes are always the largest as cooling proceeds. To control nucleation systematically, the upper size needs to be controlled. Because biological nucleators are also structurally diverse, and widely reported in various freezing systems, the genes for INAs are known and have already been expressed heterologously (Lee 1990). We can predict that in future their industrial application can only expand. Even now, the technological value of nucleators are expressed in scientific and patent literature and some examples are:

(i) pest control, by deliberate ice seeding of supercooled insects (Lee 1997);
(ii) rapid test for *Salmonella*, by incorporating INA genes into specific bacteriophages (Worthy 1990);
(iii) enhanced freeze concentration, by incorporating nucleators in the freezing mixture (Kumeno *et al.* 1994);
(iv) accelerated freezing of foods, with claimed energy savings by removal of supercooling (Li *et al.* 1997); and
(v) facilitated freeze drying, by increasing ice crystal sizes in the presence of INA actives (Watanabe & Arai 1987).

Whether these processes are active commercially is unlikely at present, but there is little doubt that nucleators will find uses in higher added value industries.

7. WHERE NEXT?

We can see from the above that the 'ice industries' are established on fundamental principles of solution physics, but are increasingly stimulated as the science of natural cryobiology becomes converted to technologies in existing industries. This is an ideal situation for success, where science push meets technology pull.

Furthermore, biotechnology now provides the access route to molecular species that biology has evolved with high specificity. The requirement of freeze tolerance triggers the production of gene products directly associated with ice 'management' such as nucleators and antifreezes. We know that there is a host of COR genes, the functions of which are not yet known. Many of them probably do not produce proteins so directly involved with ice, but control metabolism which prepares organs and tissue for the stress of ice formation, osmotic dehydration and freeze concentration. All of these secondary products could be produced in large quantities once their functions are elucidated. For example, the possible stabilization of synthetic membranes by lectin and cryoprotectin (Hincha *et al.* 1990, 1997) and the modification of cell wall properties by a variety of enzymes are obvious candidates with potential technological value.

Apart from that, discovering the elegance with which evolution has solved the problems of coping with the cold provides a fascinating challenge.

REFERENCES

Ablett, S., Izzard, M. J. & Lillford, P. J. 1992 Differential scanning calorimetric study of frozen sucrose and glycerol solutions. *J. Chem. Soc. Faraday Trans.* **88**, 789–794.

Birdseye, C. & Hall, B. 1931 Refrigerating apparatus. US patent no. 1822123.

Blanshard, D. M. V. & Lillford, P. J. 1993 *The glassy state in foods*. Nottingham University Press.

Chen, T. H. H., Burke, M. J. & Gusta, L. V. 1995 Freezing tolerance in plants: an overview. In *Biological ice nucleation and its applications* (ed. R. E. Lee Jr, G. J. Warren & L. V. Gusta), pp. 115–136. St Paul, MN: APS Press.

Clemmings, J. F., Huang, V. T., Rosinwald, D. R. & Zoerb, H. F. 1996 Frozen compositions with antifreeze protein additives. Patent no. WO9639878.

Cole, B. A., Levine, H. I., McGuire, M. T., Nelson, K. J. & Slade, L. 1983 Soft, frozen dessert formulation. US patent no. 4374154.

Cutler, A. J., Saleem, M., Kendall, E., Gusta V, L., Georges, F. & Fletcher, G. L. 1989 Winter flounder antifreeze protein improves cold hardiness of plant tissues. *J. Plant Physiol.* **135**, 351–354.

Duman, J. G., Wu, D. G., Lu, X., Tursman, D. & Olsen, T. M. 1991 Adaptation of insects to subzero temperatures. *Q. Rev. Biol.* **4**, 387–410.

Duncum, S. N., Edwards, A. R., Gordon, K. R., Holt, C. B., Lips, A. & Osborne, C. G. 1994 Hydrate inhibition. Patent no. WO9425727.

Finney, D. J. 1976 Soft ice-cream. US patent no. 3993793.

Franks, F., Auffret, A. D. & Aldous, B. J. 1996 Compositions in glassy phase stabilised by a sugar. Patent no. WO9633744, Pafra Ltd.

Grandum, S. & Nakagomi, K. 1997 Characteristics of ice slurry containing antifreeze proteins for ice storage applications. *J. Thermophys. Heat Transfer* **11**, 461–466.

Hew, C. L., Fletcher, G. L. & Davies, P. J. 1995 Transgenic salmon: tailoring the genome for food production. *J. Fish Biol.* **47**, 1–19.

Hightower, R., Baden, C., Pentes, E., Lund, P. & Dunsmuir, P. 1991 Expression of antifreeze proteins in transgenic plants. *Plant Mol. Biol.* **17**, 1013–1021.

Hincha, D. K., Heber, U. & Schmitt, J. M. 1990 Proteins from frost hardy leaves protect thylakoids. *Planta* **180**, 416–419.

Hincha, D. K., Pfuller, U. & Schmitt, J. M. 1997 The concentration of cryoprotective lectins in mistletoe. *Planta* **203**, 140–144.

Koushafar, H., Pham, L., Lee, C. & Rubinsky, B. 1997 Chemical adjuvant cryosurgery with antifreeze proteins. *J. Surg. Oncol.* **66**, 114–121.

Kumeno, E., Kurimoto, K., Nakahama, N. & Watanabe, M. 1994 Functional properties of freeze concentrated egg white foam. *Biosci. Biotechnol. Biochem.* **58**, 447–450.

Lee, R. E. 1997 Method for destroying insect pests. US patent no. 5622698.

Lee, R. E., Warren, G. I. & Crusta, L. V. 1995 *Biological ice nucleation and its application*. New York: APS Press.

Lee, T.-C. 1990 Production of frozen foods and other products. Patent no. EP0397781.

Li, J., Izquierdo, M. & Lee, T.-C. 1997 Effects of ice nucleation active bacteria on the freezing of some model food systems. *Int. J. Food Sci. Technol.* **32**, 41–49.

Lillford, P. J. & Holt, C. B. 1994 Antifreeze proteins. *J. Food Engng* **22**, 475–482.

Needham, D., Smallwood, K. & Fenn, R. A. 1998 Frozen food products especially ice-cream containing antifreeze peptides. Patent no. WO9804146.

Owen, L. B., Masterson, D. M. & Green, S. J. 1987 Rapid construction of ice structures with chemically treated sea water. US patent no. 4637217.

Payne, S. R., Sandford, D., Harris, A. & Young, O. A. 1994 The effect of antifreeze proteins on chilled and frozen meat. *Meat Sci.* **37**, 429–438.

Roser, B. J. 1987 Protection of proteins and the like. Patent no. WO870016, Quadrant Bioresources Ltd.

Roser, B. J. 1989 Preservation of viruses. Patent no. WO8906542, Quadrant Bioresources Ltd.

Rubinsky, B., Arav, A. & DeVries, A. L. 1992 The cryoprotective effect of antifreeze peptides from Antarctic fishes. *Cryobiology* **29**, 69–79.

Rudolph, A. S., Crowe, J. H. & Crowe, L. S. 1986 Effects of stabilizing agents proline, betaine, and trehose on membrane phospholipids. *Arch. Biochem. Biophys.* **245**, 134–143.

Stein, T. M., Gordon, S. H. & Greene, R. V. 1999 Amino acids as plasticizers. II. Use of quantitative structure–property relationships to predict the behaviour of monoammoniummonocarboxylate plasticizers in starch–glycerol blends. *Carbohydr. Polymers* **39**, 7–16.

Storey, K. B. & Storey, J. M. 1988 Freeze tolerance in animals. *Physiol. Rev.* **68**, 27–84.

Tunnacliffe, A. G., Welsh, D. T., Roser, B. J., Dhaliwal, K. S. & Colaco, C. 1998 Methods of preserving procaryotic cells. Patent no. WO9824882, Quadrant Holdings Cambridge Ltd.

Uemura, M. & Steponkus, P. 1997 Effect of cold acclimation on the lipid composition of the inner and outer membrane of the chloroplast envelope isolated from rye leaves. *Plant Physiol.* **114**, 1493–1500.

Warren, G. J., Mueller, G. M., KcKown, R. L. & Dunsmuir, P. 1990 Antifreeze polypeptides. Patent no. WO9013571.

Watanabe, M. & Arai, S. 1987 Freezing of water in the presence of ice nucleating agents. *Agric. Biol. Chem.* **51**, 557–563.

Weiser, R. L., Wallner, S. J. & Waddell, J. W. 1990 Cell-wall and extensin messenger RNA changes during cold-acclimation of pea-seedlings. *Plant Physiol.* **93**, 1021–1026.

Woerpel, M. D. 1980 Snow making. US patent no. 4200228.

Worthy, W. 1990 Bacterial assay exploits ice nucleation research. *Chem. Engng News* **68**, 25–26.

Discussion

T. Haymet (*Department of Chemistry, University of Houston, Houston, TX, USA*). The picture on the right of the frozen strawberries, there are these voids presumably made by ice formation. There are at least two possibilities: one is that the ice has simply pushed the cell walls away, the other possibility is that it has destroyed cell walls, but without knowing about how this was stained or otherwise analysed it looks like they have not been pushed away. It somehow looks from that picture that they have been destroyed. Do you have any opinion on how those voids were made and which of the two possibilities was responsible?

C. B. Holt. Just looking at it, is it not the case that around the cell they are thicker, like a build-up of cell debris?

T. Haymet. I think it is a fascinating question of whether they could be destroyed or just pushed aside. I think that is probably an important question.

C. B. Holt. Yes, I have always assumed they had been pushed aside and this particular picture is new to me. Just off the top of my head I cannot think how it would actually get rid of cell walls.

J. Laybourn-Parry (*School of Life and Environmental*

Sciences, University of Nottingham, Nottingham, UK). Maggie, have you got anything to offer because you were showing us some pictures of carrots in the same mode?

M. Smallwood (*Centre for Novel Agricultural Production, Department of Biology, University of York, York, UK*). In our pictures you can very clearly see that the cells and the edges of those gaps are squashed and I think, again my ageing eyes, but you can see places where there are obviously thickened squashed bits at the edges. I do not think you need to hypothesize that the walls have just disappeared.

C. Sidebottom (*Colworth Laboratory, Unilever Research, Bedford, UK*). I think it is true that in some cases where you get ice cavities the cells have actually been pushed apart. So you get ice growing through the structure and there is cell separation. But in other cases you can see in some of the microscopy, actually fracturing of cell walls where the ice forms. So I think, depending often on the freezing rate and the way the tissue has been treated, you can get both effects.

C. B. Holt. What, the cell wall can disappear completely, broken up into little parts?

C. Sidebottom. It is because it is fractured by the ice and then actually just gets pushed back. You get fragments of cell wall that are pushed back.

E. Benson (*Division of Molecular and Life Sciences, University of Abertay, Dundee, UK*). In these types of cells you would probably get quite large vacuoles so the vacuoles themselves will cause a lot of damage obviously, once the water freezes within them. So there will be a big difference between highly meristematic cytoplasmic cells and those that are highly vacuolated.

J. Laybourn-Parry. I was interested in the AFPs because Peter's talk showed that there are now synthetic AFPs. I seem to remember a young group at lunch time talking about AFPs who said they can produce bucket loads of these synthetic AFPs. So presumably, you could get designer AFPs to make whatever texture you liked in terms of crystal shape in ice-cream. I mean, has Unilever moved into this because I know there have been problems about the idea of genetically modified food organisms to mass produce AFPs, and it seems to me that one way out of that problem, which is never going to go away really, the genetically modified organism issue, is to use synthetic AFPs.

C. B. Holt. I do not think the public would like those either: synthesized, how do you mean?

J. Laybourn-Parry. Well, organic chemists can make these now. Peter, you were showing synthetic AFPs in your talk.

P. L. Davies (*Department of Biochemistry, Queen's University, Ontario, Canada*). Yes, the synthetic AFPs were made by solid-phase synthesis but they are not made in bucket loads. We are still looking at 10 mg, 100 mg quantities. I think if you do want to make kilogram quantities of AFPs you are probably better off using plants as bioreactors. I think that is the way to go.

C. B. Holt. Yes, we have been producing them by fermentation by bucket loads. The idea of shape is interesting. Different AFPs give you different ice crystal shapes. So you can think if you wanted one specific ice crystal shape then maybe you could tailor it by either mixing together AFPs that you know about. Or, when we get to the stage, making an AFP which would actually give us the ice crystal shape. Traditionally when you freeze foods you have no control whatsoever over the ice crystals that you get, none at all. The exciting thing about AFPs was that for the very first time we could start to do something about it and not just do it with engineering but do it just by an additive put in at a low level.

M. Smallwood. I was interested in what you were saying about polymers forming glasses. A plant cell is surrounded by cell walls which are composed of complex polymers. As far as I know you do not see ice crystallizing between the plasma membrane of plant cells and the wall. There has been a lot of speculation about how modification to that cell wall may actually prevent ice propagating from outside the cell to the membrane of the cell and whether that could actually form a glass.

C. B. Holt. You mean the glass could form a barrier to the ice. Is that what you are suggesting?

M. Smallwood. I wondered.

C. B. Holt. Yes you certainly could get that effect: if you had a concentrated polymer solution and you then froze it you would end up with ice crystals embedded in a glassy matrix. You cannot get any diffusion, or not on any reasonable time-scale within the glassy matrix, so you then could not get ice one side and nucleating ice the other side, so you could have one side supercool; perhaps you could do that. Of course polymers are tricky things, they do all sorts of other things as well. But yes, it is a reasonable hypothesis.

GLOSSARY

AFP: antifreeze protein
COR: cold on regulated
INA: ice nucleating agent